

Genetic diversity analysis and parentage verification of Taishu horses using 31 microsatellites

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The Taishu horse in Tsushima is one of eight Japanese native breeds. The breed is on the verge of extinction due to a rapid decrease in numbers since the 1960s owing to motorization in Japan. In this study, we aimed to confirm the pedigree information of 52 horses by genotyping 31 microsatellites in order to avoid inbreeding. Parentage verification failed to identify genetic contradictions among trios (sires, dams, and foals) registered with the Japan Equine Affairs Association (JEAA). Pedigree information registered at the JEAA was obtained and adequately understood. Additionally, the genetic diversity of the Taishu horses was evaluated and compared with those of other Japanese native breeds. The average values for the number of alleles, observed heterozygosity, expected heterozygosity, and inbreeding coefficient were 4.7, 0.643, 0.632, and -0.02 , respectively. Using the Structure software, the 52 horses were classified into three subgroups based on the individuals with more than 50% of specific genetic components. The phylogenetic trees created based on neighbor-joining classification tended to be consistent among the stallions. The effective population size was 27.5 and lower than that required for maintaining 90% genetic variation in the source population over a period of 100 years (47.5). Compared with the other Japanese breeds, the Taishu horse population included in the current study exhibited moderate genetic diversity. Our study will contribute to reconsideration of the breeding strategy of Taishu horses.

Key words: conservation, genetic diversity, microsatellite DNA, parentage verification, Taishu horse

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Introduction

Tsushima Island is located between the northern Kyushu area and the southern area of the Korean Peninsula (Fig. 1). It is possibly one of the first places where ancient Japanese horses were bred. Tsushima has historically strong ties with horses. Nishinakagawa *et al.* [13, 14] investigated the

skeletal remains of cattle and horses from archaeological sites in Japan. They suggested that horses were introduced to Japan via the Korean Peninsula between the 2nd and 5th centuries [13, 14]. The Taishu horse in Tsushima is one of eight Japanese native breeds (Fig. 2), and it may descend from the first horse that arrived in Japan. Tozaki *et al.* identified all Japanese native horses as descendants of Mongolian horses. They reported that Taishu horses are genetically closer to Mongolian horses than to other native breeds [25]. Their findings support the hypothesis of Nishinakagawa *et al.* based on genetic analysis.

Taishu horses were used as packhorses, and over 4,000 of them were bred in Tsushima around 1900 [8]. However, their population has decreased sharply, and they are on the verge of extinction due to the increase in motorization since

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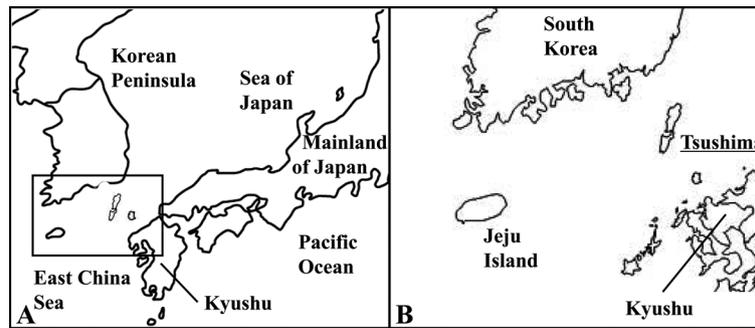


Fig. 1. Map showing the location of Tsushima Island (breeding spot of Taishu horses) in the Kyushu area of Japan. Tsushima Island comprises Tsushima City in Nagasaki Prefecture, which is in the Kyushu area of Japan (A). Tsushima Island is located between the Korean Peninsula and Kyushu (B).



Fig. 2. Physical appearance of the Taishu horse.

the 1960s. According to a statistical report by the Japan Equine Affairs Association (JEAA), the lowest ever number of Taishu horses was 27 in 2005, and since then, Tsushima City has made efforts to conserve Taishu horses. Because the last Taishu horse owner ceased breeding and handed over a mare to Tsushima City in 2021, Tsushima City is now the only organization that breeds Taishu horses in Tsushima.

Consequently, the number of Taishu horses has slightly increased, and their population in 2022 stands at 55 horses. Of them, 45 horses are within Tsushima, with the other 10 horses outside Tsushima, particularly in three zoos in different areas of Japan. However, the population size is still quite small, and Taishu horses have become a rare breed of horses. Thus, they were designated as a natural monument of Tsushima City in 2019 to promote their conservation.

Tezuka *et al.* compared the genetic diversity of the Taishu horse using single nucleotide polymorphisms (SNPs) among those living in and outside Tsushima. Their suggested mating

plan included individuals outside of Tsushima and might be an effective strategy to conserve the Taishu horse breed [24].

Microsatellites are simple sequence repeats with a higher mutation rate than SNPs [12]. Microsatellites have been used for parentage verification in Thoroughbred horse registration in Japan [9]. In conservation biology and population genetics, they are also utilized to characterize population structure and determine the genetic relationships within populations [5, 7].

In this study, we first performed a parentage verification for Taishu horses using 31 microsatellites, which have been used for the same purpose in other Japanese horses [11, 19–22]. Incorrect pedigree registration increases the risk of inbreeding. In addition, in the management of small numbers of livestock, it is important to have a complete understanding of pedigree information to avoid inbreeding. Next, the genetic diversity of Taishu horses was evaluated and compared with those of other Japanese breeds. The population sizes of all Japanese native horses have decreased since the 1960s; thus, each native breed is in a similar vulnerable situation. Comparison of the genetic diversity of Taishu horses with those of other native breeds is meaningful for our comprehension of the present genetic composition of Taishu horses.

Materials and Methods

Animals

All experimental protocols were approved by the Animal Care Committee of Gifu University (approval number: 2021-067) and performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. In this study, 52 blood samples (15 males, 29 females, and eight geldings) were collected in vacuum blood collection tubes with EDTA-2Na. The sampling for this study was conducted on two occasions, once in 2014

and once in 2021, and a total of 52 blood samples were obtained. Four old horses (two males and two females) from which we collected blood samples in 2014 have since died. The average age of the live horses was 11.6 years, ranging between 1 and 27 years.

According to the pedigree records available at the JEAA, 25 pairs of double-parent offspring and eight pairs of single-parent offspring were identified among the 52 horses.

Microsatellites and genotyping

Genomic DNA was extracted using a QIAcube Connect (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Thirty-one microsatellites (AHT4, AHT5, ASB2, ASB17, ASB23, CA425, HMS2, HMS3, HMS6, HMS7, HTG4, HTG10, LEX33, TKY19, TKY28, TKY279, TKY287, TKY294, TKY297, TKY301, TKY312, TKY321, TKY325, TKY333, TKY337, TKY341, TKY343, TKY344, TKY374, TKY394, and VHL20) were used in this study.

These microsatellites were amplified according to the protocols described by Kakoi *et al.* [9] and Tozaki *et al.* [26]. The amplified PCR products were electrophoresed using a 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Allele discrimination was performed using the GeneMapper™ 6 software (Thermo Fisher Scientific, Waltham, MA, USA) based on the nomenclature of the International Society for Animal Genetics.

Calculation of genetic statistics

The number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (F_{IS}) were calculated according to Weir and Cockerham using GENEPOP version 4.7 [16, 17, 27]. The effective population size (N_e) was calculated using the heterozygote-excess method in progeny according to the following formula [18]: $N_e = 1 / [2 (H_o - H_e) / H_e] + 1 / \{2 [(H_o - H_e) / H_e + 1]\}$.

Genetic structure analysis

To estimate population structure, cluster analysis was performed using STRUCTURE version 2.3.4 [15] based on a Bayesian analysis with five repeated runs. The population number (K) was set to between 2–10, the burn-in length was set to 10,000, and the Markov chain Monte Carlo (MCHC) number was set to 50,000. Structure Harvester [2] was used to calculate ΔK [3] and estimate the appropriate K value.

Neighbor-joining tree

The genetic distance of individuals (proportion of shared alleles, DPS) was calculated using Microsatellite Analyzer version 4.05 [1]. A neighbor-joining tree (NJ tree) was visualized using NEIGHBOR implemented in PHYLIP version 3.69 [4] and MEGA version 6.06 [23].

Table 1. Number of alleles (N_a), observed heterozygosity (H_o), and expected heterozygosity (H_e) of each allele in this study

Markers	N_a	H_o	H_e	F_{IS}
AHT4	5	0.73	0.683	-0.071
AHT5	5	0.365	0.364	-0.003
ASB2	5	0.731	0.686	-0.066
ASB17	6	0.692	0.649	-0.067
ASB23	6	0.712	0.723	0.016
CA425	4	0.712	0.688	-0.034
HMS2	5	0.75	0.771	0.027
HMS3	4	0.673	0.706	0.047
HMS6	5	0.654	0.655	0.002
HMS7	4	0.788	0.746	-0.057
HTG4	4	0.48	0.552	0.131
HTG10	4	0.788	0.688	-0.148
LEX33	5	0.769	0.688	-0.119
TKY19	4	0.288	0.261	-0.107
TKY28	4	0.615	0.653	0.058
TKY279	5	0.846	0.742	-0.141
TKY287	5	0.712	0.7	-0.017
TKY294	3	0.596	0.537	-0.112
TKY297	6	0.5	0.505	0.011
TKY301	4	0.5	0.439	-0.142
TKY312	5	0.673	0.743	0.095
TKY321	3	0.462	0.456	-0.011
TKY325	6	0.769	0.794	0.031
TKY333	6	0.654	0.701	0.068
TKY337	5	0.635	0.68	0.068
TKY341	3	0.481	0.515	0.067
TKY343	6	0.827	0.761	-0.088
TKY344	4	0.481	0.444	-0.084
TKY374	6	0.712	0.654	-0.089
TKY394	6	0.673	0.709	0.051
VHL20	4	0.692	0.705	-0.009
Mean	4.7	0.643	0.632	-0.02

Results

Genotyping and parentage verification

The microsatellites were amplified and genotyped. Based on the genotypes of 31 microsatellites, genetic contradictions regarding pedigree information were not identified among the sets (please refer to the Materials and Methods). The pedigree information registered at the JEAA was obtained and adequately understood.

Genetic diversity

Table 1 lists the N_a , H_o , H_e , and F_{IS} values. The average N_a was 4.7, and it ranged between 3 and 6. The average H_o was 0.643, and it ranged from 0.288 in TKY19 to 0.846 in TKY279. H_e was 0.632 on average, and it ranged between 0.261 in TKY19 and 0.794 in TKY325. The average F_{IS} was -0.02, and it ranged from -0.142 in TKY301 to 0.131 in HTG4. N_e was calculated to be 27.5. Table 2 lists the

Table 2. Number of alleles (N_a), observed heterozygosity (H_o), and expected heterozygosity (H_e) based on this study and those of eight Japanese horse breeds

Breed	N_a	H_o	H_e	Population size	Reference
Taishu	4.7	0.643	0.632	29♀, 15♂, 8 geldings	this study
Taishu	4.6	0.655	0.649	16	Kakoi <i>et al.</i> (2007) [10]
Yonaguni	4.4	0.591	0.601	58♀, 13♂, 7 geldings	Senju <i>et al.</i> (2017) [20]
Miyako	4.2	0.701	0.649	18♀, 17♂	Senju <i>et al.</i> (2017) [19]
Kiso	6.3	0.674	0.662	96♀, 12♂, 17 geldings	Takasu <i>et al.</i> (2012) [22]
Misaki	3	0.509	0.497	39♀, 38♂	Kobayashi <i>et al.</i> (2019) [11]
Tokara	3	0.424	0.481	123	Senokuchi <i>et al.</i> (2018) [21]
Noma	3.6	0.667	0.649	14	Kakoi <i>et al.</i> (2007) [10]
Hokkaido	6.3	0.682	0.69	28	Kakoi <i>et al.</i> (2007) [10]

♀, female; ♂, male.

N_a , H_o , and H_e values of the Taishu, Yonaguni, Miyako, Kiso, Misaki, Tokara, Noma, and Hokkaido breeds based on previous studies [10, 11, 19–22].

Genetic structure analysis

Using the Structure software, we found that the maximum ΔK (19.01) was at $K=3$. The genetic components of the 52 horses are shown in Fig. 3. The 52 horses were classified into three subgroups by individuals with more than 50% of specific genetic components (red, green, and blue).

Neighbor-joining trees

NJ trees were constructed with 52 horses (Fig. 4). When investigating the associations with four stallions (Sire A, Sire B, Sire C, and Sire D), the NJ-tree classification tended to be consistent with these stallions (Fig. 4A). In addition, the NJ-tree classification was similar to that of the major genetic components determined by cluster analysis (Fig. 4B).

Discussion

In this study, we determined the genotypes of almost all living Taishu horses and confirmed that the complete pedigree information registered with the JEAA is correct. Taishu horses are housed in individual stables, and breeding is strictly managed by Tsushima City. Therefore, it was assumed that there was no discrepancy among pedigree data.

In the past 20 years, the number of Taishu horses has increased to approximately 50. In contrast, the genetic diversity (N_a , H_o , and H_e) among them has seemingly been declining compared with that reported by Kakoi *et al.* in 2007 [10]. However, compared with seven other Japanese breeds, the population of Taishu horses included in the current study exhibited moderate genetic diversity. The inbreeding coefficient (F_{IS}) was -0.02 in this study,

so extreme inbreeding is presumed to have not occurred. Therefore, the breeding strategies pursued over the past 20 years might be beneficial and should be continued in the future. This study suggests that some modern Taishu horses possibly derived from some founders a few generations ago contributed to the genetic diversity. As Taishu horses might not be able to maintain their current genetic diversity, we should monitor this parameter in the future and compare it to the findings of previous studies.

To evaluate the genetic diversity of Taishu horses from a different perspective, we also calculated N_e , which was crucial for maintaining 90% of the genetic diversity for 100 years. It was obtained using the following formula: $N_e=475/L$, where L is the generation time, which reflects the average duration of time for a species to produce the next generation [6]. Taishu horses breed approximately between the ages of 5 and 14, so we assumed L to be 10. A generation time of 10 years for the Taishu horse suggests that the N_e required to maintain 90% genetic diversity for 100 years is 47.5. Thus, the required N_e is much larger than that observed in this study (27.5). These results suggest that the number of Taishu horses must be increased.

The NJ-tree and cluster analyses showed comparable results. In other words, the sub-clusters were shown to result from differences in stallions. The effect of four stallion lines of Taishu horses was strongly reflected in these analyses. This suggests that, in the next generation of mating, selection of different stallions lines will lead to the maintenance of genetic diversity. Given that Taishu horses comprise a small population, monitoring their pedigree information and planning their mating using different stallion lines might be prudent.

In conclusion, we confirmed the accuracy of the JEAA pedigree registry and performed a genetic diversity analysis using microsatellites. This study will be valuable in reconsidering the mating strategy for Taishu horses. Performing microsatellite analysis every few years and comparing the

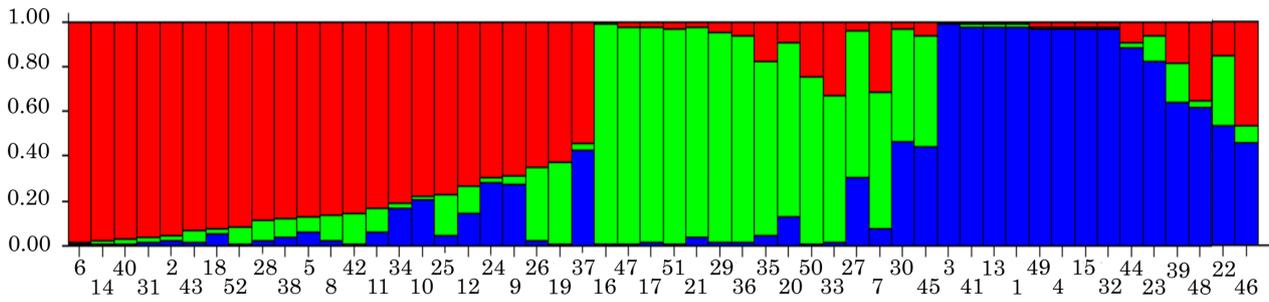


Fig. 3. Cluster analysis revealed that the Taishu horses currently have three major genetic components. They can be divided into three subpopulations based on the major components in each horse: subpopulation I (over 50% of red component), subpopulation II (over 50% of green component), and subpopulation III (over 50% of blue component). The optimal K value was estimated to be 3 ($\Delta K=19.01$), as determined by the Structure Harvester program.

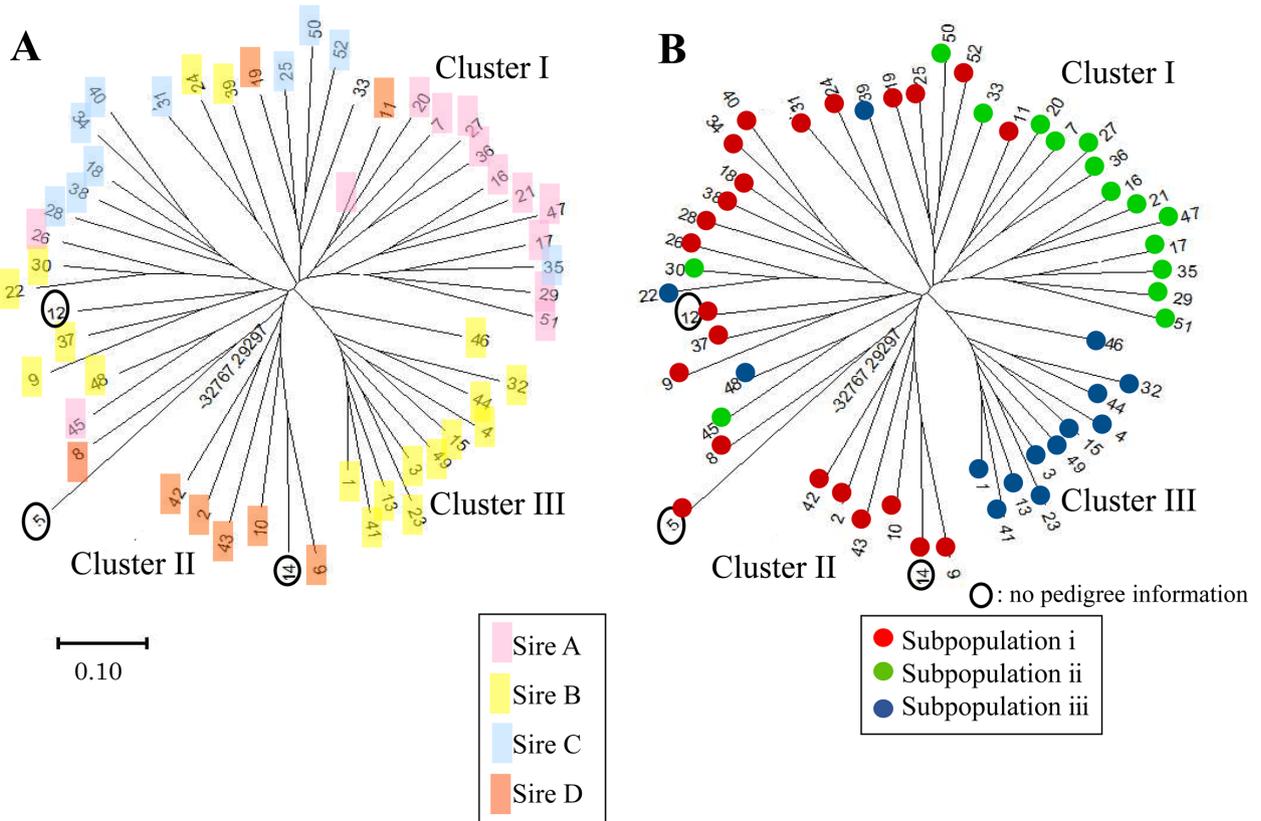


Fig. 4. Neighbor-joining trees based on genetic distances calculated from the ratio of shared alleles. Panel A: Colors show four stallion lines in Taishu horses. Pink, sire A; yellow, sire B, blue, sire C; and orange, sire D. Panel B: Various colors represent the subpopulations classified based on cluster analysis: subpopulation I (over 50% of red component), subpopulation II (over 50% of green component), and subpopulation III (over 50% of blue component; see Fig. 3).

results to previous reports is crucial in monitoring genetic diversity.

To maintain and increase genetic diversity, the roles of Taishu horses must evolve to meet current lifestyle require-

ments. While their role as packhorses might be obsolete, they could be trained to become teachers for children and companions, as well as to assist in mental health therapies. Taishu horses are calm and friendly and enjoy interactions

with people, especially children. Children learn to build and maintain relationships with other people while taking care of horses. Their calmness could be healing for people with mental health conditions.

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